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EXHIBIT B

Immunochemical Isolation and Characterization of Ovalbumin Messenger Ribonucleic Acid*

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SUMMARY

Hen oviduct ovalbumin messenger RNA has been purified to apparent homogeneity and its physical and molecular properties have been examined. Purification was achieved through the use of indirect immunoprecipitation to isolate ovalbumin-synthesizing polysomes and the use of poly(U)-Sepharose chromatography to separate quantitatively ovalbumin messenger RNA from ribosomal RNA. Ovalbumin mRNA was purified 90 to 100-fold over oviduct polysomal RNA as judged by both the rate of hybridization to a complementary DNA and by translation in a rabbit reticulocyte lysate protein-synthesizing system. Isolated ovalbumin mRNA migrates as a single sharp symmetrical peak on sucrose gradient sedimentation and polyacrylamide gel electrophoresis.

The molecular weight of ovalbumin mRNA determined by sedimentation in denaturing dimethylsulfoxide gradients is 700,000 (equivalent to 2,180 nucleotides). The complexity of purified ovalbumin mRNA determined from the relative rate of hybridization to a complementary DNA is 2,280 nucleotides. Since ovalbumin synthesis requires only 1,161 nucleotides, ovalbumin mRNA appears to contain approximately 1,150 untranslated nucleotides. The average length of the polyadenylate sequence in ovalbumin mRNA is only 44 nucleotides and it does not account for a significant fraction of the untranslated nucleotides.

The investigation of the mechanisms controlling transcription and translation in animal cells would be facilitated greatly by the possession of purified messenger RNAs coding for the synthesis of specific proteins. Recent efforts have centered on the isolation and characterization of specific messenger RNAs, and the mRNAs coding for several proteins including hemoglobin (1, 2), immunoglobulins (3–5), silk fibroin (6), and histone IV (7) have been at least partially purified and characterized. These mRNAs represent a major fraction of their cell-specific protein synthesis, and usually have been separated from total cell messenger RNA by size fractionation and from ribosomal RNA by oligo(dT)-cellulose chromatography. In this report, we describe the isolation of ovalbumin mRNA by indirect immunoprecipitation (8) and poly(U)-Sepharose chromatography; an approach that should be applicable both to those mRNAs which code for proteins representing a small fraction of cell protein synthesis and to mRNAs which contain a relatively short polyadenylate sequence.

Ovalbumin mRNA is especially favorable for investigation because it codes for synthesis of a differentiated cell product and its level is controlled by steroid hormones (9–11). By using isolated ovalbumin mRNA it is possible to investigate the effects of steroid hormones on ovalbumin mRNA synthesis, processing translation, and degradation.

EXPERIMENTAL PROCEDURE

Materials—Most of the chemicals and animals used have been described previously (8). [^{3}H]dCTP (30 Ci per mmol) was from Schwarz-Mann; oligo(dT)-cellulose and oligo(dT) (14 to 16 nucleotides in length) were from Collaborative Research. S_1 nuclease was obtained from Sigma α-amylase (10). Poly(U) was from Miles and cyanogen bromide Sepharose was from Pharmacia. The tissue press was from Harvard Apparatus Co. [^{3}H]Poly(U) (146 cpm per ng) was from New England Nuclear. Antibodies were prepared, purified, and made ribonuclease-free as described previously (8, 11).

Polysome Preparation—Oviduct polysomes were prepared as described previously (11) except that whole oviduct was minced through a ribonuclease-free tissue press just prior to homogenization. This separates the serous membrane from the oviduct without tedious scraping and reduces the time required for homogenization by approximately 50%. Polysomes were used immediately or were stored in liquid nitrogen.

Isolation of Ovalbumin-synthesizing Polysomes—Indirect immunoprecipitation of ovalbumin-synthesizing polysomes was carried out essentially as described previously (8) except that 17 to 25 A_{260} units of polysomes per ml were incubated with 145 μg of purified rabbit anti-ovalbumin per ml. The antibody-nascent chain-polysome complex was precipitated by incubation with goat anti-rabbit immunoglobulin for 2 hours at 0°. Increasing the concentration of polysomes from 10 to approximately 20 A_{260} units per ml slows formation of the insoluble complex and necessitates increasing the incubation time from 1 to 2 hours.

Poly(U)-Sepharose Chromatography—RNA was deproteinized and washed as described previously (8, 12). Poly(U)-Sepharose was synthesized essentially as described by Firtel and Lodish (13). RNA was dissolved in buffer containing 10 mM Hepes,[1] pH 7.4, 250 mM NaCl, 5 mM EDTA, and 1% sodium dodecyl sulfate (total sodium ion concentration of 0.3 M) and was applied to a poly(U)-

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[1] The abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Sepharose column (0.6 × 2 cm). The column was washed with 30 ml of the above buffer followed by 30 ml of the same buffer containing only 50 mM NaCl (Na^+ = 100 mM). Ovalbumin mRNA was eluted with 70% formamide containing 1 mM Hepes, pH 7.4, and 1 mM EDTA. To precipitate the eluted mRNA, 2 volumes of water and sodium acetate were added to a final concentration of 150 mM. After the addition of 2.5 volumes of ethanol, the RNA was precipitated overnight at −20° and was collected by centrifugation.

Quantitation of Ovalbumin mRNA—Rabbit reticulocyte lysate assays were carried out exactly as described previously (8, 14). Complementary DNA was synthesized from purified ovalbumin mRNA as described (10, 15). Hybridization was at 68° in 300 mM NaCl, 10 mM Tris-HCl, pH 7.1 (25°), 2 mM EDTA, 0.5 mg per ml of reticulocyte RNA, and 0.1% sodium dodecyl sulfate in a total volume of 10 μl (10). Hybrid formation was assayed using S_1 nuclease followed by precipitation with trichloroacetic acid and trapping of the hybrids on Whatman GF/C filters (10, 15).

Polyacrylamide Gel Electrophoresis—RNA electrophoresis was carried out in 2.5% acrylamide gels (16, 17). Gels were scanned at 260 nm in a Gilford spectrophotometer equipped with a linear transport device. To elute RNA from gel slices they were homogenized in a Dounce homogenizer with a loose fitting pestle and were left for 24 hours in a buffer containing 10 mM Hepes, pH 7.4, 0.15 M NaCl, and 1% sodium dodecyl sulfate. The solubilized RNA was precipitated with ethanol three times and was assayed for its ability to code for ovalbumin synthesis in a rabbit reticulocyte lysate.

Dimethylsulfoxide-Sucrose Gradients—Denaturing 0 to 8% sucrose gradients containing 99% dimethylsulfoxide, 0.25 mM Tris, pH 7.1, and 1 mM EDTA were run as described by Strauss *et al.* (18) with the modifications of Firtel and Lodish (13). Dried RNA samples were dissolved in a mixture of 12% H_2O, 33% dimethylformamide, and 55% dimethylsulfoxide and were preheated at 60° for 5 min prior to application. Sedimentation was for 18 hours at 30° in a Beckman SW 41 rotor at 40,000 rpm.

Hybridization of Ovalbumin mRNA with [^{3}H]Poly(U)—Purified ovalbumin mRNA was freed of the small amount of poly(U) which co-elutes from poly(U)-Sepharose by sedimentation on a dimethylsulfoxide gradient. Hybridization with poly(U) was by a modification of the method of Bishop *et al.* (19). Hybridizations were carried out in 25 mM Tris, pH 7.1, 5 mM $MgCl_2$ and 500 mM NaCl in a total volume of 100 μl. A 10- to 100-fold excess of [^{3}H]poly(U) was present in all reactions. Hybridizations were for 15 min at 30°. Samples were diluted to 2 ml with the above buffer, 20 μg of pancreatic RNase were added, and the samples were incubated for 15 min at 30°. Calf thymus DNA (0.2 mg) was added as carrier and nucleic acids were precipitated with cold 6% trichloroacetic acid and were trapped on Whatman GF/C filters.

RESULTS

Purification of Ovalbumin Messenger RNA—Purification of a messenger RNA entails separation from other cell mRNAs and from rRNA. Ovalbumin mRNA was separated from total oviduct mRNA by indirect immunoprecipitation of ovalbumin-synthesizing polysomes. This entails (*a*) incubating oviduct polysomes with anti-ovalbumin, resulting in the binding of antibody to nascent peptide chains on polysomes synthesizing ovalbumin; (*b*) incubation of the soluble antibody-nascent chain-polysome complex with an anti-antibody prepared against the first antibody; and (*c*) sedimentation of the insoluble antibody-antibody-polysome complex twice through a detergent containing discontinuous sucrose gradient to remove nonspecifically adsorbed material.

The best separation of ovalbumin mRNA and rRNA was achieved by poly(U)-Sepharose chromatography (Table I). Cellulose appears to have a much greater tendency to bind rRNA nonspecifically than does Sepharose[2] and the purification achieved using cellulose consequently is less than that achieved using Sepharose. The low yields on cellulose and nitrocellulose filters

[2] D. J. Shapiro, unpublished observations.

TABLE I

Fractionation of messenger and ribosomal RNA

Millipore partition was carried out exactly as described by Palacios *et al.* (12). For cellulose chromatography, RNA was dissolved in 5 ml of 25 mM Tris, pH 7.5, containing 0.5 M NaCl and was passed slowly over a column (0.8 to 10 cm) at 25°. The column was washed with 20 ml of the above buffer and then with 20 ml of the above buffer containing 0.2 M NaCl. RNA was eluted with water containing 1 mM Hepes, pH 7.5, and 0.1 mM EDTA. Unbound RNA repassed over Millipore filters or cellulose exhibited no significant binding.[3] Benzoylated DEAE-cellulose chromatography was carried out as described by Murphy and Attardi (20) and Sedat *et al.* (21). RNA for oligo (dT)-cellulose chromatography was dissolved in 2 ml of 25 mM Tris, pH 7.5, and 0.15 M NaCl, and was applied to a column (0.6 to 4 cm) at 4°. The column was washed with 30 ml of the above buffer. RNA was eluted by a temperature gradient. Most ovalbumin mRNA activity eluted at 18-25° and these fractions were pooled and used for reticulocyte lysate assays. Poly(U)-Sepharose chromatography was carried out as described under "Materials and Methods." Reticulocyte lysate assays at three RNA concentrations on the original RNA, the unbound RNA, and the eluted RNA were carried out as described under "Materials and Methods."

Fractionation method	Ovalbumin mRNA specific activity	Purification	Yield
	cpm/μg RNA	*-fold*	%
None	600		
Millipore	16,000	26	30
Cellulose	7,000	12	37
Benzoylated DEAE-cellulose	5,000	8	6
Oligo(dT)-cellulose	22,000	36	42
Poly(U)-Sepharose	35,000	58	72

suggest that although ovalbumin contains a polyadenylate sequence, its average length is relatively short. Only about one-third of the ovalbumin mRNA molecules appear to contain polyadenylate sequences, 75 to 100 nucleotides long, which are required for efficient binding to nitrocellulose filters (22) and cellulose (23).

Using indirect immunoprecipitation to separate ovalbumin mRNA from total oviduct mRNA and poly(U)-Sepharose chromatography to separate mRNA and rRNA, we have purified ovalbumin mRNA (Table II).

Purity of Isolated Ovalbumin mRNA—Ovalbumin mRNA isolated by indirect immunoprecipitation and poly(U)-Sepharose chromatography has not been fractionated with respect to size. If the isolated RNA were homogeneous in size, this would be an excellent criterion of purity. We therefore examined the size of the isolated RNA by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis.

Isolated ovalbumin messenger RNA migrates as a single sharp symmetrical peak on sodium dodecyl sulfate sucrose gradient sedimentation. Ovalbumin mRNA activity is coincident with the main optical density peak at approximately 16 S (Fig. 1). Analysis of the isolated mRNA by polyacrylamide gel electrophoresis reveals a sharp symmetrical peak migrating slightly slower than 18 S rRNA (Fig. 2). Isolated ovalbumin mRNA appears free of detectable 28 S rRNA and appears to contain at most 1 to 2% 18 S rRNA. The apparent size of ovalbumin mRNA determined by its sedimentation rate in sucrose gradients is at least 50% less than that estimated from polyacrylamide gel electrophoresis. Inasmuch as this large difference in apparent

TABLE II

Purification of ovalbumin messenger RNA

A_{260} units (1,422) of oviduct polysomes in 70 ml of 25 mM Tris, pH 7.1, 25 mM NaCl, and 5 mM $MgCl_2$ were subjected to indirect immunoprecipitation as described under "Materials and Methods." Immunoprecipitated RNA totaled 446 A_{260} units. The total activity is the product of the amount of RNA (μg) and the specific activity of the RNA. Fold purification and yield were calculated from the specific activity and total activity, respectively. Reticulocyte lysate assays were carried out at four RNA concentrations. The RNA fraction not bound to the poly(U)-Sepharose contained 23% of the mRNA activity applied whereas the bound and eluted fraction contained 71% of the applied ovalbumin mRNA activity.

Fraction	RNA	Specific activity	Total activity	Purification	Yield
	mg	*cpm/μg RNA*	*cpm × 10^{-6}*	*-fold*	%
Polysomal RNA.....	31.6	1,133	35.8	1	100
Immunoprecipitated RNA..	9.9	1,877	18.6	1.7	52
Poly(U)-Sepharose eluate..	0.12	109,907	13.4	97	38

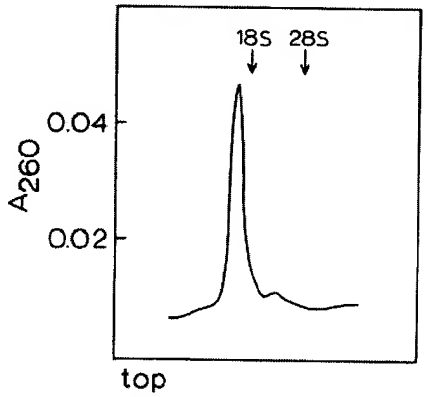

FIG. 1. Sedimentation profile of ovalbumin mRNA on sucrose gradients. Purified ovalbumin mRNA (1.8 μg) dissolved in 50 μl of 25 mM Tris, pH 7.1, containing 1% sodium dodecyl sulfate and 2 mM EDTA was layered over a linear 5 to 20% sucrose gradient and was sedimented for 4½ hours at 225,000 × *g*. Polysomal RNA markers were run in separate tubes. Gradient fractions were collected and RNA was precipitated with ethanol and was assayed for ovalbumin mRNA activity in a rabbit reticulocyte lysate. The small peak of absorbance between 18 S and 28 S rRNA probably is electronic noise and is not present in more recent sucrose gradients.

molecular weight might be due to differences in secondary structures and hydrodynamic properties of mRNA and rRNA, we determined the molecular weight of ovalbumin mRNA under conditions where secondary structure would not be a factor.

Size of Ovalbumin mRNA—RNA species appear to migrate as random polymers devoid of secondary structure and to sediment with velocities directly proportional to their molecular weights in denaturing dimethylsulfoxide gradients (18). Ovalbumin mRNA sedimented in 99% dimethylsulfoxide, 0 to 8% sucrose gradients under conditions which appear to preclude aggregates or a significant amount of secondary structure, migrates to the same point as chicken 18 S ribosomal RNA (Fig. 3). The molecular weight of chicken 18 S rRNA is approximately 700,000 (24). This corresponds to a molecule 2,180 nucleotides in length.

We next determined the size of ovalbumin mRNA by nucleic acid hybridization, a method which is independent of secondary structure and sedimentation rate and does not even require an

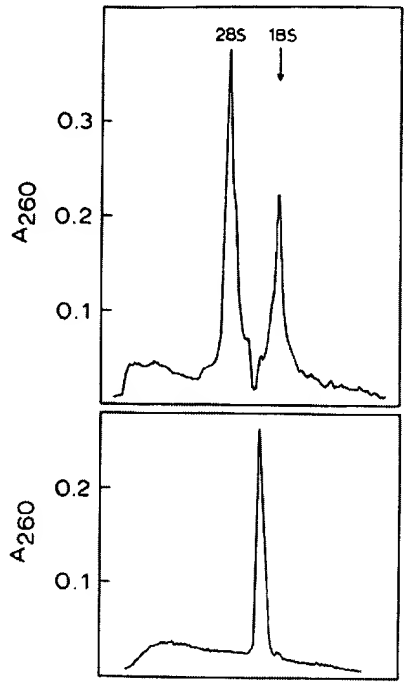

FIG. 2. Acrylamide gel electrophoresis of purified ovalbumin mRNA. Ten micrograms of oviduct polysomal RNA and 4 μg of purified ovalbumin mRNA were dissolved in 25 μl of electrophoresis buffer containing 10% glycerol and bromphenol blue. Preelectrophoresis of the 2.5% acrylamide gels (14 × 0.6 cm) was for 45 min. The gel was scanned after electrophoresis in a Gilford spectrophotometer with a linear transit. Background A_{260} peaks near the bottom of the gel and trails off. The background is not due to RNA because it is found on blank gels following electrophoresis. After scanning, the gel was sliced and RNA was eluted as described under "Materials and Methods" and was assayed for ovalbumin mRNA activity, which was exclusively localized under the major optical density peak.

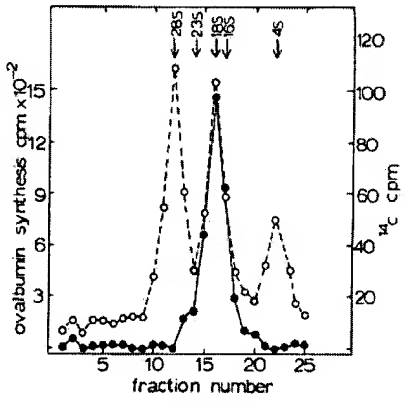

FIG. 3. Sedimentation of ovalbumin mRNA in denaturing dimethylsulfoxide gradients. ^{3}H-Labeled hen oviduct RNA (1,200 cpm per μg) and 0.2 μg of purified ovalbumin mRNA were dissolved in a mixture of water, dimethylformamide, and dimethylsulfoxide (13, 18) heated to 60° for 5 min and cooled rapidly. Sedimentation on 99% dimethylsulfoxide 0 to 8% sucrose gradients was for 18 hours at 30° and 270,000 × *g*. Fractions were collected and diluted with 2 volumes of water containing 0.2 M sodium acetate and RNA was precipitated with ethanol. The RNA was redissolved and an aliquot was counted to locate the labeled chick RNA (O- - -O). The remainder of each fraction was assayed for ovalbumin mRNA activity (●——●). *Escherichia coli* 16 S and 23 S rRNA markers were sedimented in parallel gradients. The *E. coli* optical density profiles are omitted for simplicity.

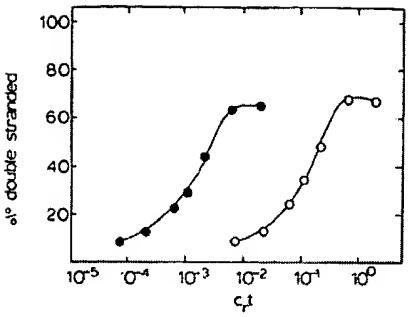

FIG. 4. Kinetics of reassociation of purified ovalbumin mRNA and oviduct polysomal RNA with complementary DNA. Purified ovalbumin mRNA (0.00048 A_{260} per 10 μl hybridization) (●) or total oviduct polysomal RNA (0.048 A_{260} per 10 μl hybridization) (○) was mixed in 10 μl with 300 cpm of single-stranded complementary DNA (10^4 cpm per ng) prepared against purified ovalbumin mRNA (see "Materials and Methods"). Hybridization was at 68° for 0 to 24 hours, and hybrids were scored as described under "Materials and Methods." The background obtained by counting a blank filter is subtracted from all values. Continuing the hybridization for an additional 4 days did not appreciably increase the per cent of cDNA hybridized.

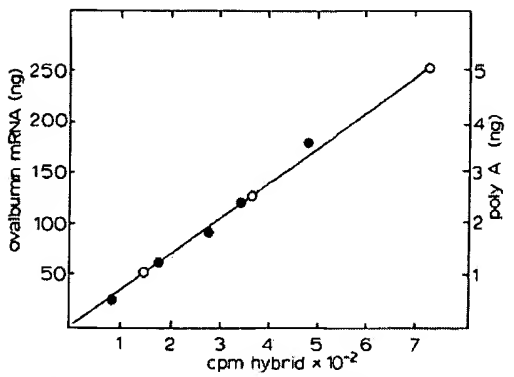

FIG. 5. Hybridization of ovalbumin mRNA with [^{3}H]poly(U). Purified ovalbumin mRNA (●) or poly(rA) (○) in the indicated amounts was annealed with [^{3}H]poly(U) (146 cpm per ng). Hybridization, ribonuclease digestion, and trapping of the hybrids were carried out as described under "Materials and Methods."

intact mRNA molecule. Within broad limits, the rate of hybridization appears to be independent of RNA complexity[3] in these RNA excess RNA-DNA hybridizations (25). The rate of hybridization of ovalbumin mRNA to its complementary DNA is proportional to the number of ovalbumin sequences in a given amount of mRNA. Hybridization of total oviduct polysomal RNA and purified ovalbumin mRNA to complementary DNA prepared against purified ovalbumin mRNA is shown in Fig. 4. Purified ovalbumin mRNA hybridizes with a $C_rt^1_{1/2}$ of 1.43×10^{-3} compared to a $C_rt_{1/2}$ of 1.29×10^{-1} for polysomal RNA. Purification of ovalbumin mRNA thus results in a 90-fold increase in the number of ovalbumin mRNA sequences. This is in excellent agreement with the 97-fold increase in specific ovalbumin-synthesizing activity obtained by *in vitro* translation (Table II). The complexity of ovalbumin mRNA can be calculated by comparison to the $C_rt_{1/2}$ and the complexity of poliovirus RNA.

$$\frac{\text{complexity ovalbumin mRNA}}{\text{complexity poliovirus RNA}} = \frac{C_rt_{1/2}\text{ ovalbumin mRNA}}{C_rt_{1/2}\text{ poliovirus RNA}}$$

Poliovirus RNA has a complexity of 7,500 nucleotides (26) and a $C_rt_{1/2}$ under our hybridization conditions of 4.7×10^{-3} (15). The complexity of ovalbumin mRNA is 2,280 nucleotides which corresponds to a molecular weight of 730,000. This is in good agreement with the value of 2,180 nucleotides obtained from dimethylsulfoxide gradients (Fig. 3). The minimum size of ovalbumin mRNA is 1,161 nucleotides (ovalbumin contains 387 amino acids), approximately 1,150 nucleotides less than actually appear in ovalbumin mRNA. We next determined the fraction of these 1,150 nucleotides which is due to the polyadenylate sequences in ovalbumin mRNA.

Determination of Length of Polyadenylate Sequence in Ovalbumin mRNA—Ovalbumin mRNA binds to nitrocellulose filters, oligo-(dT)-cellulose, and poly(U)-Sepharose (Table I), suggesting the presence of a polyadenylate sequence. The average length of this polyadenylate sequence was determined by quantitative hybridization with [^{3}H]poly(U). Poly(rA) forms a double-stranded A-U hybrid under these hybridization conditions[2] (19). Purified ovalbumin mRNA contains 2.0% poly(A) (Fig. 5), and each 2,200 nucleotide mRNA molecule therefore contains a poly(A) sequence with an average length of 44 nucleotides. Of course, the possibility that the poly(A) is present in several small sequences rather than in a single sequence is not excluded by this experiment.

DISCUSSION

Isolation of ovalbumin mRNA entails separation from total oviduct mRNA and from rRNA. Separation of ovalbumin mRNA from total oviduct mRNA was achieved by indirect immunoprecipitation of ovalbumin-synthesizing polysomes with anti-ovalbumin and anti-antibody. Quantitation of the amount of nonspecific precipitation and of the purity of the precipitated mRNA has been described in detail in a previous paper (8). Measurements of the extent of nonspecific precipitation and the yield of immunoprecipitated RNA indicate that the isolated ovalbumin mRNA is approximately 99% pure with respect to contamination by other species of mRNA. Briefly, reaction of oviduct polysomes with anti-bovine serum albumin results in precipitation of only 0.4% of the ovalbumin-synthesizing polysomes. Isolation of rat liver albumin-synthesizing polysomes from a mixture of hen oviduct and rat liver polysomes demonstrated that only 0.4% of the ovalbumin-synthesizing polysomes and mRNA was nonspecifically precipitated with the albumin-synthesizing polysomes (8). Ovalbumin mRNA isolated by indirect immunoprecipitation thus appears to be essentially free of contamination by other species of mRNA.

Ovalbumin mRNA and rRNA were quantitatively separated by poly(U)-Sepharose chromatography. The extent of the contamination of purified ovalbumin mRNA by rRNA was examined by the display of the isolated mRNA on sucrose gradients (Fig. 1) and acrylamide gels (Fig. 2). A sharp symmetrical peak containing the ovalbumin mRNA is observed on both sucrose gradients and acrylamide gels. The 28 S rRNA is undetectable and the isolated mRNA contains at most 1 to 2% 18 S rRNA (Fig. 2).

Additional proof that the isolated ovalbumin mRNA which is 90- to 97-fold purified is free of contaminants can be obtained by calculating the purification expected for an mRNA with a molecular weight of 700,000.[4] Ovalbumin mRNA comprises approxi-

[3] Complexity is defined as the number of nucleotides necessary to code for the unit of information in a population of nucleic acid molecules. C_rt is the product of concentration of RNA (in moles of nucleotides per liter) and time (in seconds). $C_rt_{1/2}$ is the C_rt (in moles·s per liter) at which the hybridization of a given population of nucleic acid molecules is half completed.

[4] Assuming ovalbumin-synthesizing polysomes contain 12 to 14 ribosomes and are half of the total polysomes (27), the fraction of ovalbumin mRNA in the total polysomal RNA and the fold puri-

mately 1.1% of oviduct polysomal RNA and purification should result in an 88-fold enrichment[4] in good agreement with the 90- to 97-fold observed for ovalbumin mRNA. Ovalbumin mRNA isolated by indirect immunoprecipitation and poly(U)-Sepharose chromatography appears to be approximately 98% pure with respect to contamination by rRNA and other mRNAs.

Assay of ovalbumin mRNA at various stages of purification permits us to calculate the yield of mRNA, which is approximately 40% (Table II). This is a minimum estimate because any inactivated ovalbumin mRNA which co-purified would not be considered. In most purifications of mRNA repeated size fractionation and oligo(dT)-cellulose chromatography result in low yields of mRNA. Indirect immunoprecipitation and poly-(U)-Sepharose chromatography permit isolation of ovalbumin mRNA in good yield and should prove widely applicable to the isolation of mRNAs.

We have purified ovalbumin mRNA approximately 95-fold over oviduct polysomal RNA by measuring specific ovalbumin-synthesizing activity by an *in vitro* translation assay (Table II) and approximately 90-fold by measuring relative numbers of ovalbumin mRNA sequences using hybridization to complementary DNA (Fig. 4).

In previous investigations Palacios *et al.* (12) achieved a 40-fold purification of ovalbumin mRNA by immunoadsorption and partition on nitrocellulose filters. O'Malley and his colleagues (28) report a 100-fold purification from total oviduct nucleic acid (equivalent to about 50-fold from polysomal RNA). Haines *et al.* (27) recently have purified ovalbumin mRNA 67-fold by size fractionation and oligo(dT)-cellulose chromatography. All of these preparations contain other mRNAs or rRNA. The molecular weight of ovalbumin mRNA and the length of the polyadenylate sequence were not determined accurately in these studies.

The purification of ovalbumin mRNA clearly does not result in progressive inactivation of ovalbumin mRNA as the fold purification measured by translation (an activity measure) and hybridization which measures only amount is very similar. Inasmuch as the proportion of active ovalbumin mRNA sequences is constant through purification and the immunoprecipitation requires mRNA which is in polysomes containing nascent peptide chains, the proportion of purified ovalbumin mRNA which is active should be very high. In contrast, Haines *et al.* (27) found that only about half of the ovalbumin mRNA molecules isolated from total polysomal RNA by size fractionation were active.

The apparent molecular weight of ovalbumin mRNA on sucrose gradients and acrylamide gels varies widely (approximately 550,000 and 850,000). To eliminate the possibility that differences in secondary structure of mRNA and rRNA produce these differences, the molecular weight of ovalbumin mRNA was determined under denaturing conditions. Purified ovalbumin mRNA and 18 S chick rRNA co-migrate on denaturing dimethylsulfoxide gradients indicating a molecular weight of 700,000 (equivalent to 2,180 nucleotides). An independent determination of molecular weight was made by comparison of the rate of hybridization of ovalbumin mRNA and its cDNA to that of poliovirus and its cDNA. This method is independent of mRNA structure and does not even require that the ovalbumin mRNA be intact. Inasmuch as secondary structure and aggregation artifacts are eliminated, hybridization kinetics seems a useful adjunct to methods based on the mobility and migration of RNA. The molecular weight determined by hybridization is 730,000, in excellent agreement with the value of 700,000 from dimethylsulfoxide gradients. Ovalbumin mRNA thus appears to contain approximately 2,220 nucleotides. Ovalbumin mRNA need contain only 1,161 nucleotides to code for ovalbumin which contains 387 amino acids.

Ovalbumin mRNA appears to contain about 1,150 untranslated nucleotides. Inasmuch as ovalbumin mRNA contains a poly(A) sequence, its contribution to the untranslated nucleotides was assessed. By quantitative poly(U) hybridization, we have determined the average length of the polyadenylate sequence to be 44 nucleotides. The ovalbumin mRNA molecule is nearly large enough to code for the synthesis of 2 molecules of ovalbumin. This is most improbable, as antibody binding studies have demonstrated that the ovalbumin-synthesizing polysome contains 12 to 14 ribosomes (11) which is the size expected of a polysome synthesizing a protein with a molecular weight of 40,000 to 45,000. There is no evidence that ovalbumin is synthesized as a high molecular precursor either in oviduct (8, 11, 12) or in a rabbit reticulocyte lysate primed with oviduct RNA (14). Ovalbumin mRNA contains approximately 1,100 untranslated nucleotides with no known function. Most other eukaryotic mRNAs also appear to contain a significant proportion of untranslated sequences. Immunoglobulin (29), α-crystallin (30), and histone (31) mRNAs all appear to be significantly larger than required to code for the proteins' amino acid sequence.

The large untranslated sequence in ovalbumin mRNA may contain the much smaller sequences located at or near the 5'-OH terminus of eukaryotic mRNAs which hybridize to middle repetitive DNA (13, 32, 33). The potential role of such sequences both in regulating synthesis and in the cytoplasmic function of ovalbumin mRNA is currently under investigation.

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fication expected can be calculated

$$\frac{\text{molecular weight of ovalbumin mRNA}}{\text{molecular weight of rRNA + mRNA}} \times 50\% \frac{0.7 \times 10^6}{13(0.7 + 1.6) \times 10^6 + 0.7 \times 10^6}$$

$$\times 50\% = 1.14\% \quad \text{or} \quad \frac{100}{1.14} = 88\text{-fold}$$

The fold purification of ovalbumin mRNA determined by hybridization is:

$$\frac{C_r t_{1/2} \text{ polysomal RNA } 1.29 \times 10^{-1}}{C_r t_{1/2} \text{ ovalbumin mRNA } 1.43 \times 10^{-3}} = 90$$

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